Inactivation of Enzymes in an Aqueous Solution by Micro-bubbles of Super critical Carbon Dioxide

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Received August 12, 1994

Enzyme solutions of glucoamylase, acid protease, alkaline protease and lipase were treated with micro-bubbles of supercritical carbon dioxide (SC CO₂) fed from a cylindrical filter nozzle. The micro-bubbles of SC CO₂ could increase the CO₂ concentration in the sample solution from 0.4 to 0.92 mol/l at 25 MPa and 35°C, and hence could improve the efficiency of inactivation by about 3 times compared to treating without the filter nozzle. Alkaline protease and lipase in the solution could be completely inactivated by the treatment at 35°C and 15 MPa for 30 min. With the inactivation of glucoamylase and acid protease, their residual activity-CO₂ density profiles consisted of two straight lines with intersections at density values of 0.82 and 0.60 g/cm³, respectively. These enzymes showed an abrupt decrease in activation in regions above the CO₂ density of their intersections.

Supercritical fluids generally have a similar density to liquids and a similar viscosity to gases, therefore, they have a solvent power similar to that of liquids, but with mass transfer characteristics like those of gases. Among the supercritical fluids, supercritical carbon dioxide (SC CO₂) has several advantages such as its non toxicity, non flammability, no residual chemical problem, and low to moderate operating temperature and pressure over other fluids, and is the most widely used solvent for supercritical fluid extraction (SFE). This process has recently received considerable attention by the food industry, there being several applications for the extraction of vegetable oils from various oil seeds. Nevertheless, the application of SC CO₂ has been limited to food products with a low water content because the efficiency of SFE with liquid foods was insufficient. We have already reported the technique of micro-bubble SC CO₂ extractions, which could yield a high recovery of volatile compounds dissolved in an aqueous solution.

Apart from the direct exploitation of the dissolving capacity of SC CO₂, reviews of the influence of SC CO₂ on proteins, enzymes and amino acids have recently been published. Taniguchi et al. have studied the retention of activity of x-amy lase, glucose oxidase, lipase, and catalase under SC CO₂ circumstances. However, the study only dealt with enzyme preparations whose water content was 5–7 wt%.

Balaban et al. have investigated the inactivation of pectinesterase in orange juice by SC CO₂. While Arreola et al. have reported the effects of SC CO₂ on some quality attributes of orange juice. We also consider that it is important for the food industry to investigate the effects of SC CO₂ treatment on enzymes dissolved in an aqueous solution: i.e., active enzymes remaining in food products that result in unpleasant changes and degrade their quality during storage or distribution.

This present study was carried out to investigate the effect of SC CO₂ treatment on the activity of glucoamylase, acid protease, lipase and alkaline protease in an aqueous solution. It was considered that the concentration of SC CO₂ in the solution could be a most important factor for inactivating enzymes, so we applied the technique of micro-bubble SC CO₂ treatment.

Materials and Methods

Enzyme solutions. Acid protease from Aspergillus oryzae (optimum pH 3.0 and 50°C temperature), alkaline protease from Bacillus subtilis (pH 8.0 and 60°C), glucoamylase from Rizopus delemar (pH 4.5–5.5 and 50°C), and lipase from Rizopus japonicus (pH 7.0 and 40°C), each of which could be responsible for degrading food quality, were purchased from Nagase Seikakaku Kogyo Co., Ltd. These enzymes were dissolved in deionized water to a concentration of 20–100 mg/100 ml, and the enzyme solutions were used for the SC CO₂ treatment.

Apparatus and procedure for SC CO₂ treatment. The SC CO₂ treatment (SCT) was carried out using a Milton Roy X-10 system (Riveria Beach, FL). This system consisted of a 120-ml treatment vessel, which has a cylindrical filter made of sintered stainless steel for feeding SC CO₂ in micro-bubbles, temperature controllers, pressure gauges, and two plunger-type pumps. The desired pressure of CO₂ was set by a back-pressure regulating valve, while the temperature of the treatment vessel was controlled to within ±1.0°C with an electrical heater and temperature controller.

For each experiment, around 100 ml of an enzyme solution was loaded into the treatment vessel and had been preheated to the experimental temperature. Air in the vessel was purged with CO₂, and then pressurized by CO₂. The temperature and pressure being maintained at the experimental levels. The treatment conditions were varied in the ranges of pressure from 4 to 25 MPa and temperature from 35 to 50°C. Until the pressure reached the experimental level. CO₂ was fed at 4.0 g/min for about 10 min, and feeding was stopped. At the end of SCT for 30 min, the vessel was depressurized by slowly operating the pressure regulating valve over a period of about 5 min.

Determination of CO₂ concentration in the solution during SCT. The CO₂ concentration in the enzyme solution during SCT was measured after delivering about 10 ml of the pressurized enzyme solution directly delivered into 20 ml of 1 M NaOH solution in a closed system. This alkaline solution that absorbed CO₂ was weighed and titrated with 0.5 M HCl against phenolphthalein and methyl orange.

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Measurement of enzymic activity
Acid protease and alkaline protease assay. About 1 ml of each enzyme solution was added to 1.5 ml of 2% casein in McIlvaine buffer (pH 3.0) or to a 0.05 M sodium phosphate buffer (pH 8.0) and then incubated at 37°C for 60 min. Each reaction was terminated by adding 3 ml of a 5% TCA solution, before the solution was filtered. To 1 ml of the filtrate was added 1 ml of a phenol reagent and the absorbance at 665 nm was measured. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine per 60 min under the assay conditions.

Lipase assay. The enzyme solution (1 ml) was added to 5 ml of an emulsion of olive oil and 4 ml of McIlvaine buffer (pH 7.0), and the mixture incubated at 37°C for 60 min. The reaction was terminated by adding 30 ml of 90% ethyl alcohol. The resulting solution was titrated with 0.05 M NaOH against phenolphthalein. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of oleic acid per 60 min under the assay conditions.

Glucose assay. The enzyme solution (1 ml) was added to 1 ml of a starch solution and 0.2 ml of a 0.2 M acetic acid buffer solution (pH 5.0), and the mixture incubated at 40°C for 20 min. The reaction was terminated by adding 0.1 ml of 1 N NaOH and neutralized by 0.1 ml of 1 N HCl. The amount of glucose formed during the reaction was determined with a glucose assay kit (Wako Pure Chemical Industry), one unit of enzyme activity being defined as the amount of enzyme required to liberate 1 mg of glucose per 60 min under the assay conditions.

Residual activity. The residual activity of the enzymes was calculated by the following formula:

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\text{Residual activity} = \frac{\text{Activity (unit/ml) of the SCT enzyme solution}}{\text{Activity (unit/ml) of the enzyme solution prepared}} \times 100
\]

Results and Discussion
Effect of filter pore size on CO₂ concentration during SC CO₂ treatment
Balaban et al. have described that an SC CO₂ treatment (SCT) is based on the hypothesis that high-pressure CO₂ dissolves in water to produce carbonic acid, thereby temporarily lowering the pH value and inactivating enzymes. Moreover, Weder et al. have shown that SCT could decrease the L-arginine content in a solution, and they proposed that, under a lower pH environment, protein-bound arginine could easily interact with CO₂, forming a bicarbonate complex. Thus, they suggested that the inactivation of enzymes by SCT could be achieved by pH-lowering and bicarbonate-forming caused by CO₂ dissolved in water. Therefore, prior to our experiments on the inactivation of enzymes, we determined the concentration of CO₂ dissolved in the sample solution during SCT and aimed to increase it by using a cylindrical micro-filter.

The concentration of CO₂ in the enzyme solution during SCT with or without the micro-filter is shown in Fig. 1. In this experiment, SCT was carried out at 35°C and 25 MPa. The result indicated that the concentration of CO₂ was significantly influenced by the way CO₂ was fed and increased with decreasing pore size of the filter. The highest concentration (about 1.1 mol/l) was achieved by using a micro-filter with a minimum pore size of 10 μm. It was desirable to use a filter of even smaller pore size to increase the CO₂ concentration, but one smaller than 10 μm was not available. The dashed line in Fig. 1 indicates the saturated level (1.4 mol/l) of CO₂ under these experimental conditions (25 MPa and 35°C), which was calculated from interpolating the Kuenen coefficient, γ = 32.59. After bubbling for 2 h, the concentration was reached 1.35 mol/1, 93% of the saturated level.

Effect of the filter on glucoamylase inactivation
The residual activity of glucoamylase after SCT at 25 MPa and 35°C with or without the filter (10 μm pore size) is shown in Fig. 2. Under these conditions, the residual activity was 17.5% when using the filter, which is about one third of that by treating without a filter. This result shows that the use of a micro-pore filter significantly influenced the inactivation of the enzymes. Thus, it was important for inactivating an enzyme to increase the concentration of SC CO₂ in the solution. The subsequent experiments were all

![Fig. 1. Effect of Filter Pore Size on CO₂ Concentration during the Supercritical CO₂ Treatment at 25 MPa and 35°C.](image)

![Fig. 2. Effect of Filter on the Residual Activity of Glucoamylase during the Supercritical CO₂ Treatment at 25 MPa and 35°C.](image)

![Fig. 3. Residual Activity of Alkaline Protease and Lipase after a Supercritical CO₂ Treatment at 35°C.](image)
carried out while using this filter.

**Inactivation of alkaline protease and lipase**

The residual activity of alkaline protease and lipase after SCT at 35°C is shown in Fig. 3. Both the enzymes were completely inactivated at a pressure above 15 MPa. On the other hand, the residual activity of alkaline protease was about 8% after incubating under atmospheric conditions at 35°C in a MacIlvaine buffer (pH 3.0; Fig. 4). Therefore, the main effect of alkaline protease inactivation could have been pH-lowering by dissolved CO₂, although the components of the buffer solution might have had some influence on the stability of the enzyme. In contrast, lipase retained about 80% of its activity after being incubated under the same conditions (Fig. 4). It appears that lipase must have been inactivated by a different mechanism from that for alkaline protease.

**Inactivation of glucoamylase and acid protease**

SCT of glucoamylase and acid protease required higher pressure and some heating in order to reduce their residual activity. Figures 5-a and 5-b show the residual activity of glucoamylase and acid protease after SCT. Only SCT with heating resulted in a great decrease in enzyme activity, SCT at 50°C and 25 MPa leading to the residual activity of glucoamylase and acid protease being 0% and 0-10%, respectively. Incubation at pH 3 under atmospheric conditions, only resulted in a little inactivation (Figs. 5-a and 5-b) while treatment at 4 MPa and 35°C also inactivated the enzymes to less than 5% (data not shown). On the other hand, Suzuki and Taniguchi have described that protein denaturation caused by a hydrostatic effect occurred only when the pressure was above 1000–3000 atm. Therefore, we consider that factors other than pH, temperature, pressure, and surface tension at the CO₂-water interface must have contributed to the enzyme inactivation.

**Effect of SC CO₂ density on the inactivation of glucoamylase and acid protease**

The effect of SC CO₂ density on the inactivation of glucoamylase and acid protease is shown in Fig. 6 with varying treatment temperature. With both enzymes, a higher density resulted in a lower residual activity, while their residual activity–density profiles consisted of two intersecting straight lines. In the case of glucoamylase, the intersection occurred at a density of 0.82 g/cm³ at all three treatment temperatures. On the other hand, acid protease had an intersection at about 0.60 g/cm³. These results show that a certain CO₂ density was needed to cause an abrupt decrease in the activity. We considered that inactivation could be caused by the sorption of CO₂ into enzyme molecules, and that the solubility of SC CO₂ toward enzyme molecules would be mainly dependent on the conformational character of the enzyme and on the CO₂ density. Hence, there would be a turning point on the profile of the inactivation or solubility at a different density for the enzymes. In addition, an increase in CO₂ concentration by micro-bubbling SC CO₂ would greatly increase in the

![Fig. 4. Residual Activity of Alkaline Protease and Lipase after Incubating in a MacIlvaine Buffer (pH 3.0) at 35°C.](image)

![Fig. 6. Effect of CO₂ Density on Inactivation of the Enzymes. Symbols: ● temperature was kept at 35°C; ○ temperature was kept at 40°C; ▲ temperature was kept at 45°C.](image)
amount of CO₂ sorption. The adsorption of SC CO₂ to protein molecules has been confirmed by a gravimetric method. A sharp peak was observed at just above the critical pressure on the adsorption isotherms for SC CO₂ with several proteins. The adsorption of SC CO₂ by dry protein molecules (water content of 3.4–8.4 wt%) would not be comparable to the absorption of SC CO₂ into enzymes in a solution. However, a similar mechanism for protein molecule–SC CO₂ interaction might be considered, and we are now investigating the inactivation mechanism of these enzymes.

References